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14. ABSTRACT

Data from our lab and others indicate that normal breast cells do not express the erythropoietin receptor (EpoR); conversely, breast cancer (CaBr) cells express functional EpoR. Expression of EpoR appears greatest in poorly oxygenated tumor regions and in patients with negative estrogen receptor status, a sign of more aggressive disease. Additionally one study demonstrated that the EpoR gene is overexpressed in patients with micrometastatic disease. The differential expression of EpoR between normal and cancerous breast cells has led us to hypothesize that acquisition of EpoR expression by mammary epithelial cells may be part of malignant transformation and may impact disease progression and metastasis. Our data, demonstrating changes in mammary epithelial cell biology associated with acquisition of EpoR expression, support this hypothesis and suggest that EpoR acquisition results in mammary epithelial cell with a premalignant phenotype. These data will help us better understand the oncogenic process in CaBr and may suggest the need for caution in administering Epo to (at least some) CaBr patients.

15. SUBJECT TERMS
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INTRODUCTION

Normal mammary epithelial cells/cell lines do not express the erythropoietin receptor (EpoR), while breast cancer (CaBr) cells - and cell lines - are EpoR-positive (1-3). Neither the functional significance (4-6) of EpoR on these nonhematopoietic cells nor that of their differential expression on normal vs. malignant mammary epithelial cells is understood. In most other instances where EpoR are found on tumor cells/cell lines, it is found on corresponding normal cells as well; thus, the mammary epithelial/CaBr model presents a unique opportunity to investigate the functional significance of the EpoR on nonhematopoieotic cells.

Erythropoietin (Epo) has widespread clinical application in the treatment of breast cancer (CaBr), where it has been demonstrated to relieve disease- or treatment-related anemia and fatigue, to improve cognitive function, and to decrease tumor/tissue hypoxia. However, several recent clinical trials have reported (7-9) that Epo treatment of at least some cancer patients (including CaBr patients) may be associated with decreased overall survival. Concerns over these and related matters have caused the FDA Oncologic Drugs Advisory Committee (ODAC) to convene two meetings – in May 2004 and again in May 2007 – to address potential safety issues associated with the use of Epo and other erythropoiesis stimulating agents (ESAs) in the oncology setting.

Taken together, available data have led us to hypothesize that EpoR acquisition 1) may be part of the process or malignant transformation for mammary epithelial cells, and 2) the EpoR may be functioning like an oncogene for mammary epithelial cells. Our working hypothesis in the current grant is that **acquisition** of EpoR by mammary epithelial cells may influence the oncogenic process and that Epo may be a growth and survival factor for CaBr cells. To test our hypothesis our original grant application proposed to insert the human EpoR into human mammary epithelial cells and, conversely, to downregulate the EpoR in human CaBr cells. The effect of EpoR acquisition (or loss) is then assessed by a series of gain- or loss-of-function studies.

BODY

We started our investigation by screening multiple human breast cancer (CaBr) cell lines, available in our laboratory, for the presence of EpoR. The cell lines that we selected varied in their *in vitro* and *in vivo* growth characteristics, as enumerated below (**Table 1**).

Table 1. **Characteristics of breast cancer cell lines used in this study.**

Cell line	Tumorigenicity *in vivo*	Invasiveness *in vitro*	Phenotype/ genotype	Morphology on plastic	ER/PgR	other
MCF-7	poorly (estrogen dependent)	weakly	luminal epithelial-like	polygonal	+/+	typical epithelial markers
T-47D	poorly (estrogen dependent)	weakly	luminal epithelial-like	polygonal	+/+	typical epithelial markers
SK-BR-3	yes	weakly	weakly luminal epithelial-like	clusters of weakly attached cells	-/-	amplified *ERBB2*
MDA-MB-231	highly	highly	stromal/ mesenchemal	fibroblastoid	-/-	over-express vimentin
Hs578T	yes (semisolid medium)	highly	stromal/ mesenchemal	fibroblastoid	-/-	over-express vimentin

We confirmed, by RT-PCR, that all of the CaBr lines we had selected express the gene for the human EpoR (**Figure 1**). Based upon the RT-PCR results, we concluded, therefore, that MCF7 (lane 3) would be a representative line to use as our EpoR-positive CaBr cell line. We further confirmed that the "normal" (nontumorigenic) mammary epithelial cell line, MCF10A (lane 4), did NOT express EpoR and would, therefore, be suitable as our EpoR-negative mammary epithelial cell line.

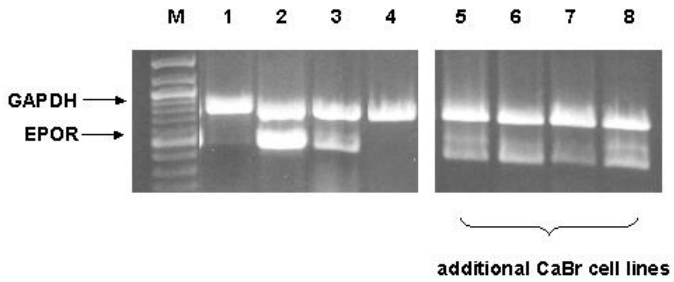

Figure 1: RT-PCR amplification of a 485 bp fragment of the human EpoR gene from human breast cancer cell lines. RNA was prepared from all cells using TriZol reagent, according to manufacturer' methods and used for subsequent RT-PCR co-amplification of EpoR and GAPDH fragements. Lane 1: BaF3 pro-B cells, which lack EpoR (negative control); lane 2: BaF3/EpoR cells, transfected with the full-length human EpoR gene (positive control); lane 3: **MCF7** (EpoR positive) CaBr cell line; lane 4: **MCF10A** (EpoR negative) mammary epithelial cells; lanes 5-8: additional (EpoR positive) CaBR cell lines – SKBR, MDA-MB231, Hs578T, and T47D, respectively.

Task 1: Downregulation of EpoR in human breast cancer cells, using antisense technology.

a. Transfection of pcDNA3.1/EpoR antisense construct into MCF7 CaBr cells. As proposed in the original grant application, we used pcDNA3.1/EpoR(AS) (pcDNA3.1$^{(-)}$ vector, containing the full length human EpoR cDNA inserted in the "antisense orientation" - i.e. the orientation that would result in transcription on EpoR "antisense message"), to transfect MCF7 cells. DOTAP (Roche) lipid-mediated transfection was utilized. Control cells were transfected with empty pcDNA3.1$^{(-)}$ vector. This method for downregulation of gene expression using full-length "antisense cDNA" was selected because of our previous success with the method (and with the particular construct) (10).

b. Single cell cloning and expansion of clonal MCF7/EpoR↓ populations. Following G418 selection and growth of G418 resistant cells, single-cell cloning resulted in the growth of numerous clones. Forty-eight robust clones were selected for further growth and expansion. These cells have been designated MCF7/EpoR↓, to designate cells with downregulated EpoR.

c. Analysis to confirm downregulation of EpoR expression in MCF7/EpoR↓ cell populations. Two-step RT-PCR, with gene-specific primers, was utilized to determine 1) how many of the selected MCF7/EpoR↓ clones contained the full-length antisense (AS) EpoR sequence and 2) how many of the MCF7/EpoR↓ clones that were successfully transfected with the AS construct had decreased levels of EpoR expression. Our survey showed that 41 of the 48 clones contained the full-length AS construct.

As documented in our Annual Report (August 2006), a problem that we have encountered with our original strategy of using the full-length EpoR AS cDNA construct is that since the AS and sense strands are of the same length, it has been extremely difficult to determine, by RT-PCR, whether

downregulation of EpoR (sense strand) expression has occurred. We have been able to demonstrate the *presence* of AS in the cells, by selecting primers that encompass [portions of] the vector sequence as well as the EpoR coding sequence. However, we have not been able to devise a primer set that would be specific for the sense strand (i.e. the actual cDNA for sense and antisense constructs has the identical sequence – only their orientation is reversed) that would allow strand-specific amplification. We have made two attempts at real-time and semi-quantitative RT-PCR to detect strand specific message (i.e. downregulation of EpoR expression) without success. Because MCF7 cells are not Epo-*dependent* (although they are Epo-responsive – *see below*) there is some subjectivity in assessing whether and/or to what degree we may have downregulated EpoR expression in our clones.

In discussion with colleagues in the laboratory we devised two potential solutions to this problem of positively identifying EpoR-downregulated clones. The first employs an EpoR AS construct that is "tagged", so it would not amplify with primers from sense sequence. The second involves the use of si/sh RNA, rather than a full-length AS sequence, for EpoR gene downregulation. In the current year we have decided to proceed with the shRNA approach, as described below.

Table 2: shRNA strategy for EpoR downregulation in MCF7 cells.

Clone	TRC ID	Target Sequence (5'-3')	Target Region* (in bp)
pLKO-EpoR1	TRCN0000058313	CGTGTCATCCACATCAATGAA	539-559
pLKO-EpoR2	TRCN0000058314	CCCTTATGAGAACAGCCTTAT	1597-1617
pLKO-EpoR3	TRCN0000058315	CACCTAAAGTACCTGTACCTT	1487-1507
pLKO-EpoR4	TRCN0000058316	TGCCAGCTTTGAGTACACTAT	1399-1419
pLKO	N/A	No insert	N/A
pLKO-NTC	N/A	CAACAAGATGAAGAGCACCAA	Non-targeting

* The target regions of pLKO-EpoR are the nucleotide numbers corresponding to EpoR mRNA sequence (NM_000121). All four pLKO-EpoR constructs target the coding region (137-1663 bp) of EpoR mRNA. N/A; not-applicable.

Using the targeted sequences, above, we recently (within the last month) have generated what we believe to be the first clones of MCF7 cells with downregulated EpoR expression (designated MCF7/EpoR^{kd}, to distinguish them from the MCF7/EpoR↓ clones that we generated using the antisense technology (**Figure 2)**. We have not been able to complete further functional analysis of these new clones prior to the expiration of the grant period.

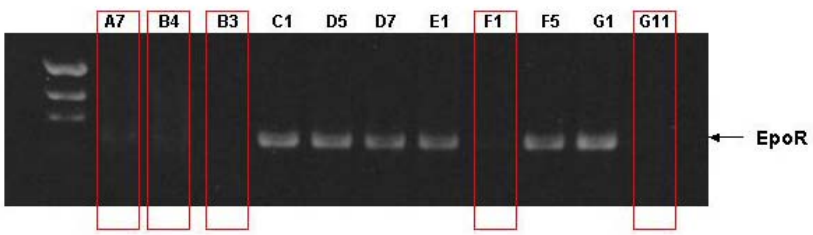

Figure 2: RT-PCR of selected MCF7/EpoR^{kd} clones resulting from shRNA knockdown of the EpoR gene in MCF7 cells.

d. Establishment of subpopulations of chronically Epo-treated MCF7 and MCF7/EpoR↓ cells. Populations of MCF7 cells have been cultured continuously in the presence of either 2 or 5 U rhEpo/ml for periods > 2 mo, to generate chronically Epo-treated cells. Similarly, we generated chronically Epo-treated counterparts for our (original) uncloned MCF7/EpoR↓ cells. Because of the problems associated with generating and verifying clonal populations of MCF7/EpoR↓ cells (as described above), and the delay in production of (new) MCF7/EpoR^{kd} clones, the generation of chronically Epo-treated (new) MCF7/EpoR^{kd} was delayed and was not completed by the end of the grant period.

Task 2: Preparation of clonal cell populations of MCF10A mammary epithelial cells expressing the full-length human EpoR (MCF10A/EpoR cells).

a. Transfection of a pcDNA3.1/EpoR into MCF10A mammary carcinoma cells. As proposed in the original grant application, we used pcDNA.3.1/EpoR (pcDNA3.1$^{(+)}$ vector, containing the full length human EpoR cDNA as insert) to transfect (EpoR negative) MCF10A cells with the human EpoR gene. As with the antisense cDNA transfection discussed above, DOTAP lipid-mediated transfection was used for each of the three separate transfections we performed. Control cells were transfected with empty pcDNA3.1$^{(+)}$ vector. 600 ug G418/ml was applied as a selection reagent.

b. Single cell cloning and expansion of MCF10A/EpoR clonal populations. Our first attempt at single-cell cloning met with failure, as the nearly 100 positive wells were lost due to fungal contamination or our incubator. Our second single-cell cloning yielded 51 stable G418-resistant clones, representing MCF10A/EpoR cell populations.

c. Analysis to confirm presence of EpoR gene and protein expression by the cells. We utilized one-step RT-PCR, with gene-specific primers, to amplify a 485-bp fragment of the EpoR gene from (uncloned) MCF10A/EpoR cells derived from two independent transfectants (**Figure 3**). This demonstrates that we were successful in introducing the EpoR gene into MCF10A cells.

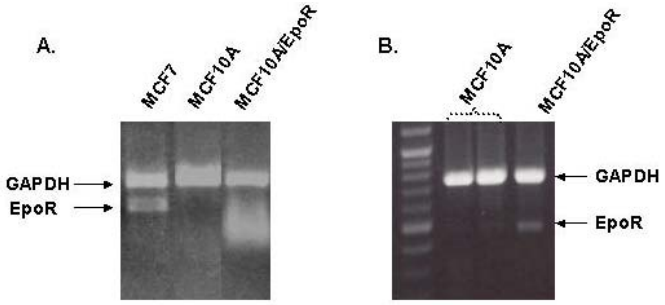

Figure 3: RNA was prepared from (EpoR+) MCF7 cells, from (EpoR-) MCF10A cells, and from two independently-generated preparations of MCF10A/EpoR cells (using TriZol reagent and following manufacturer's recommendations). EpoR -specific primers were used to amplify a 485 bp fragment of the human EpoR gene (a 797 bp fragment of the GAPDH gene was co-amplified as loading control and as a measure of RNA integrity). Panel A (left): The EpoR gene fragment is amplified from MCF7 cells (left lane) and from MCF10A/EpoR (right lane), but not from MCF10A cells (middle lane). Panel B (right): A 485 bp EpoR gene fragment is amplified from the MCF10A/EpoR transfectants (right lane), but not from either of two samples of MCF10A cells (middle two lanes).

Interestingly, we noted (**Figure 4**) that acquisition of EpoR expression by MCF10A/EpoR cells appears to induce the expression of the Epo gene as well. This is a novel and rather unexpected finding which we believe strongly supports our working hypothesis that **acquisition** of EpoR by mammary epithelial cells may influence the oncogenic process and that Epo may be a growth and survival factor for CaBr cells. We hypothesize that a functional Epo – EpoR axis may help promote cancer cell survival, potentially through an autocrine/paracrine growth-regulatory mechanism.

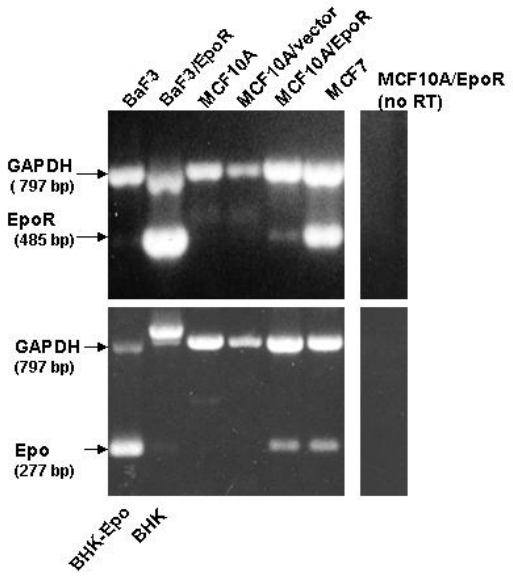

Figure 4: RT-PCR reveals that MCF10A/EpoR cells express the same size EpoR fragment as do MCF7 breast cancer cells (top) – see also Figure 3 above. Interestingly, cells transfected with EpoR (i.e. MCF10A/EpoR cells) also express the gene for Epo (bottom). Neither MCF10A nor MCF10A/vector cells express either Epo or EpoR.

d. Preliminary analysis of functionality of EpoR in MCF10A/EpoR cells. To reiterate, our hypothesis is that **acquisition** of EpoR expression by mammary epithelial cells may be related in some way to the oncogenic process in CaBr. Therefore, following transfection of MCF10A cells with the EpoR (MCF10A/EpoR cells), we began to test the effect of EpoR acquisition on the *in vitro* biology of the cells. These are preliminary experiments, and the results are those optained using uncloned MCF10A/EpoR (i.e. a mixed population of MCF10A cells, expressing varying levels of EpoR).

One of our initial observations is that MCF10A/EpoR cells appear to grow in culture more rapidly than do the "parental" MCF10A cells (**Figure 5**).

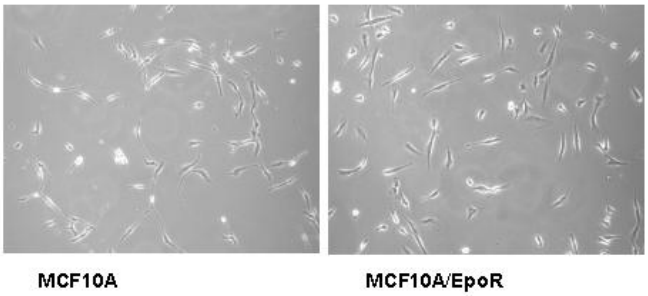

Figure 5: Equal numbers of (parental) MCF10A [left] and (transfected) MCF10A/EpoR [right] were plated in standard growth medium. Culture dishes were observed after 48 hr. The qualitative observation from this initial experiment is that the growth rate of MCF10A/EpoR is faster than that of the parental MCF10A cells.

We observed (**Figure 6**) that MCF10A/EpoR cells had altered morphology in culture compared to either "parental" MCF10A cells or MCF10A cells transfected with vector alone (MCF10A/vector cells). MCF10A (or MCF10A/vector) cells appear spindle-shaped under our culture conditions, while MCF10A/EpoR exhibit the more rounded (and, on confluence, more "cobblestone") morphology of MCF7 CaBr cells. We have not, at this juncture, further analyzed these morphological changes.

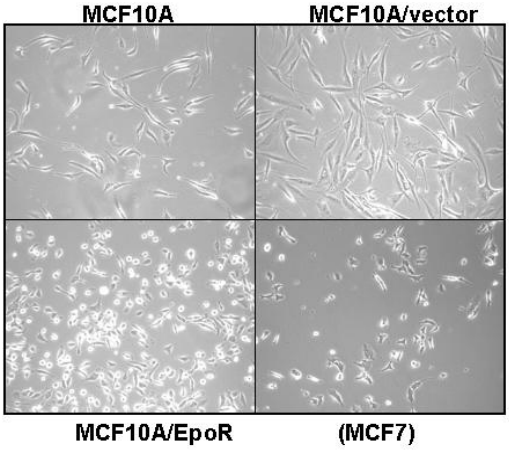

Figure 6: MCF10A/EpoR cells (bottom, left) proliferate more rapidly in culture and undergo morphological changes relative to either "parental" MCF10A or MCF10A/vector (top, left and right, respectively). MCF7 breast cancer cells are shown lower right for comparison (*note:* the plating density of MCF7 was lower than that of the other cells in this figure).

Next, we demonstrated (**Figure 7**) that MCF10A/EpoR cells appear to acquire growth-factor independence with continued passage in culture. Note that while MCF10A/EpoR appears not to require Epo for growth (nor does it require insulin or epidermal growth factor, as do parental MCF10A cells), the cells are Epo-responsive.

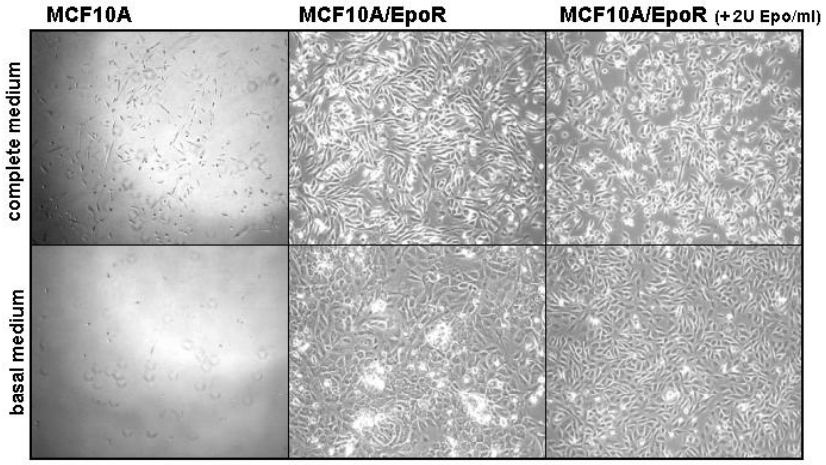

Figure 7: "Parental" MCF10A (left) and MCF10A/EpoR cells were maintained either in complete (DMEM/F12 – 5% FBS with EGF, insulin and hydrocortisone; top) or in basal (DMEM/F12 - 5% FBS; bottom) medium. MCF10A cells have an absolute requirement for growth factors for *in vitro* survival and growth, while MCF10A/EpoR proliferate robustly, even in the absence of exogenous growth factors. The addition of 2U rhEpo/ml to basal culture medium further enhanced the growth of MCF10A/EpoR cells (bottom, right).

Further data supporting the functionality of EpoR in MCF10A/EpoR cells is discussed as part of **Task 3**, below.

e. Establishment of subpopulations of MCF10A and MCF10A/EpoR in the continuous presence of Epo (chronically Epo-treated cells). MCF10A and (uncloned) MCF10A/EpoR cells were cultured for > 2 months in the presence of 2 or of 5U rhEpo/ml, to obtain chronically Epo-treated cells.

Task 3: *In vitro* studies to determine how gain or loss of EpoR expression affects the growth and survival of mammary epithelial and CaBr cells. Because of our difficulties (see Task 1) in demonstrating successful knockdown of EpoR in MCF7/EpoR↓ cells, we have had to adjust some of our experimental plan for Task 3. We have only just completed successful demonstration of EpoR downregulation using our alternative approach (shRNA knock-down), as opposed to the "antisense" approach that we had proposed (and tried) originally. The overall aim has remained the same – i.e. to demonstrate that alterations in EpoR expression affect cell growth and survival – but the reagents with which we have had to work are slightly different.

Therefore, consistent with the completion of this Task, we report our experimental data

a. Effect of Epo treatment on the proliferation of MCF10A and MCF10A/EpoR cells in liquid culture.

We conducted several experiments in which we investigated the effect of *acute* Epo exposure on the proliferation of MCF10A/vector (=MCF10A), MCF10A/EpoR and MCF10A/EpoR^{+2} cells, using an MTT assay. 48-hr exposure of cells to 0 – 1 U Epo/ml resulted in no significant proliferative response by any of the cells (**Figure 8**).

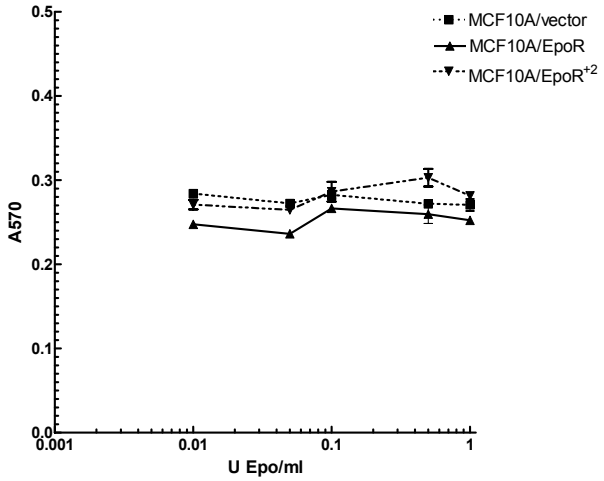

Figure 8: MCF10A/vector (=MCF10A), MCF10A/EpoR and MCF10A/EpoR^{+2} cells were washed and suspended in RPMI-5% FBS, 3000 cells/well, in a 96-well plate. Cells were incubated for 48-hr in the presence of 0-1 U rhEpo/ml, and cell proliferation was measured by an MTT assay. There is no significant effect of Epo on cell proliferation, at least under these conditions.

In contrast, we saw something rather different when we investigated the effect of *chronic* Epo treatment on the survival of MCF10A, MCF10A/EpoR and MCF10A/EpoR^{+2} cells. Cells were plated as above (i.e. 3000 cells/well in a 96-well plate, RPMI-5% FBS medium) in the absence of presence of increasing doses of paclitaxel for 48-hr (**Figure 9**). Cell survival was measured by an MTT assay.

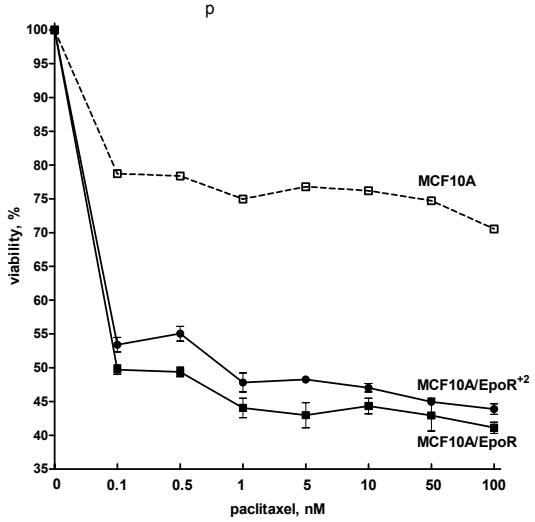

Figure 9: Cells, as indicated, were incubated in the absence or presence of paclitaxel (0 – 100 nM) for 48-hr, and cell viability (survival in presence of given dose paclitaxel / survival in absence of paclitaxel) was measured by an MTT assay. Paclitaxel acts by inhibiting microtubule growth and preferentially affects rapidly-growing cells. Data from a recent preliminary experiment indicate that MCF10A/EpoR cells (closed squares) are sensitive to paclitaxel; and that passage of MCF10A/EpoR cells for 2-mo in the presence of 2UrhEpo/ml results in cells (designated MCF10A/EpoR^{+2}) with slightly increased resistance to paclitaxel (closed circles). By comparison, the slower-growing MCF10A mammary epithelial cells (open squares) have limited sensitivity to paclitaxel over the time of this experiment.

b. Effect of Epo treatment on the growth of MCF10A and MCF10A/EpoR in semi-solid medium (soft agar culture). We also wanted to determine whether acquisition of EpoR affected colony formation in soft agar. It has been established that MCF7 cells form colonies in soft agar, and our initial experiment, shown below, suggests that Epo stimulates the growth of MCF7 soft agar colonies, by increasing the number of colonies formed (**Figure 10**). This is, to our knowledge, the first demonstration of the effect of Epo on MCF7 colony formation. MCF10A cells do not form colonies in soft agar, and our results confirm this. This experiment demonstrates that MCF10A/EpoR cells form colonies in soft agar and that the growth (size/number) of these colonies is stimulated by rhEpo.

If, in fact, *in vitro* soft agar colony-forming ability of cells is a potential model for their (predicted) *in vivo* tomorigenicity, then this novel observation may be particularly significant. The data suggest that EpoR acquisition "transforms" cells (i.e. MCF10A) that do not form colonies in soft agar and that are non-tumorigenic into cells that are anchorage-independent, form small colonies in soft agar, and are Epo-responsive. Future *in vivo* studies will be necessary to determine whether these MCF10A/EpoR cells may potentially be tumorigenic.

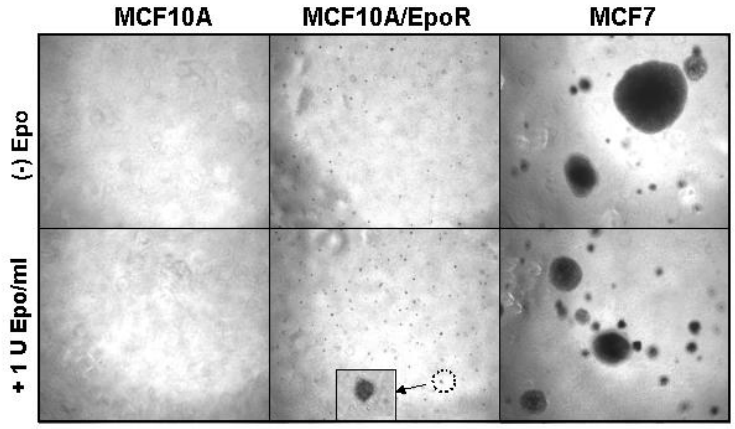

Figure 10: MCF10A cells(left) are anchorage dependent for growth – i.e., they fail to form colonies in soft agar. Conversely, MCF7 breast cancer cells (right) are anchorage independent for *in vitro* growth. MCF10A/EpoR cells (middle) appear to exhibit anchorage-independent growth, forming small colonies in soft agar. The addition of rhEpo to culture of MCF10A/EpoR cells increases the number of soft agar colonies that are formed.

c. Demonstration of signaling in response to Epo in MCF7 cells vs. MCF7/EpoR↓ cells. As started previously, we were unable to demonstrate EpoR (gene) downregulation in MCF7/EpoR↓ cells, and we are just now succeeding in demonstrating EpoR downregulation in the newly-engineered MCF7/EpoR^{kd} cells. Therefore, we have not (yet) been able to complete this sub-section of Task 3.

d. Demonstration of signaling in response to Epo in MCF10A and MCF10A/EpoR cells. We have performed preliminary signal transduction experiments, measuring the phospho-Akt and and phosphor-Erk1/2 responses of MCF10A, MCF10A/EpoR and (for comparison) MCF7. Those data are shown in **Figure 11**.

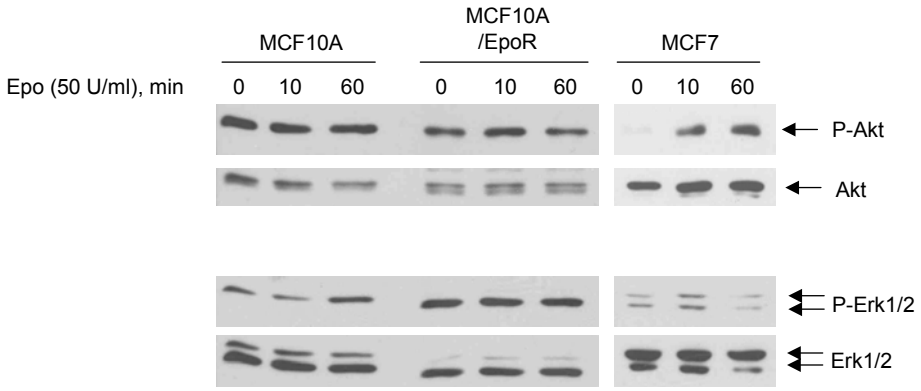

Figure 11. Epo-dependent signal transduction in MCF10A, MCF10A/EpoR and MCF7 cells. Cells were incubated with 50 U rhEpo/ml for the indicated time and total cell lysates were probed for phospho-Akt or phospho-Erk1/2. The same membrane was stripped and reprobed for total Akt or total Erk1/2 to demonstrate equal protein loading. MCF7 cells showed Epo-dependent increases in phospho-Akt (peak at 60 min)and phospho-Erk1/2 (10 min), while MCF10A or MCF10A/EpoR cells did not show a significant response under these conditions.

e. Co-culture experiments. We were not able to complete this subsection of Task 3, as we were required to spend more time than we originally had anticipated in generating and verifying our new cell constructs. We have cultured the cells in soft agar, with and without Epo (see Figure 10) but we have not yet optimized conditions for the co-culture with endothelial cells.

Key Research Accomplishments

The following points summarize the accomplishments from this grant:

- We confirmed that MCF10A cells express neither EpoR nor Epo genes, and that MCF7 CaBr cells express both EpoR and Epo.
- We introduced the human EpoR gene into EpoR-negative MCF10A mammary epithelial cells, and we demonstrated that EpoR acquisition results in concomitant/subsequent expression of the Epo gene in MCF10A/EpoR cells.
- We have demonstrated that acquisition of EpoR gene expression by MCF10A cells (=MCF10A/EpoR cells) results in morphological changes and in cells that grow faster in culture, even in the absence of exogenous erythropoietin.
- We demonstrated that while short-term exposure to exogenous Epo has no significant effect on cell proliferation in MCF10A/EpoR cells in liquid culture, chronic exposure to Epo (=MCF10A/EpoR^{+2} cells) affects cell survival.
- We demonstrated that EpoR acquisition by MCF10A cells (=MCF10A/EpoR cells) results in cells that grow in soft agar (= become anchorage independent), and that exposure of MCF10A/EpoR cells to Epo in semi-solid medium increases both the size and number of soft agar colonies.

- We have provided what we believe to be the first reported evidence that Epo augments the growth of MCF7 colonies in soft agar culture. Growth in soft agar is often reported to be an in vitro measure of (potential) *in vivo* tumorigenicity of the cells, suggesting that Epo may increase the growth potential of MCF7 tumors in *in vivo* models.
- We originally used antisense technology to downregulate EpoR gene expression in MCF7 cells (=MCF7/EpoR↓ cells). Due to problems associated with verifying the EpoR downregulation, we switched from antisense technology to shRNA technology. This has enabled us to demonstrate effective downregulation/knockdown of EpoR in MCF7 CaBr cells.
- We examined, preliminarily, the signaling response to Epo of MCF10A/EpoR cells.

Reportable results

The following are the direct result of research funded by this grant:

- We have established 41 initial clonal lines of MCF7 breast cancer cells with downregulated EpoR (=MCF7/EpoR↓) by using antisense technology and 3 new stable clonal populations of MCF7 with EpoR knocked down by shRNA (=MCF7/EpoR^{kd}).
- We have prepared chronically Epo-treated MCF7 and MCF7/EpoR by treating cells continuously in culture with either 2 or 5 U rhEpo/ml.
- We have established of two independent derivatives of MCF10A mammary carcinoma cells expressing the human EpoR (=MCF10A/EpoR) and have established clonal derivatives.
- We have established subpopulations of MCF10A/EpoR cells that have been chronically Epo-treated with either 2 or 5 U rhEpo/ml(=MCF10A/EpoR$^{+2U\ Epo/ml}$ and MCF10A/EpoR$^{+5U\ Epo/ml}$) and have begun to compare the Epo-treated and untreated cells in culture.
- We have applied for continued research funding (two grants) based upon this work and upon our working hypothesis that the EpoR may function as an oncogene in breast cells.
- We have presented an abstract at the 2007 American Association for Cancer Research annual meeting, in Los Angeles, CA. (abstract and poster attached, in Appendix).

Conclusion

We successfully have introduced the EpoR gene into (nontumorigenic, EpoR negative) MCF10A cells and have peroformed gain-of-function studies aimed at determining how EpoR acquisition affects the in vitro biology of MCF10A/EpoR cells. We have determined that EpoR acquisition results in Epo expression, and this suggests that an autocrine/paracrine mechanism of growth modulation may be operative in MCF10A/EpoR cells (similar to what we believe is operative in MCF7 CaBr cells). We hope to continue and expand these studies, with the aim of determining the effect of EpoR acquisition on the cells and, potentially, on tumorigenesis.

We have used both "antisense technology" and shRNA to downregulate EpoR expression in MCF7 CaBr cells. Initially we experienced great technical difficulty in verifying EpoR downregulation by antisense, which resulted in our switching to shRNA technology instead. We have now demonstrated to our satisfaction that we can knock down the EpoR in CaBr, and we have begun to investigate loss-of-function studies.

The reagents that we have prepared and now have at our disposal – MCF10A cells, MCF10A/EpoR, MCF10A/EpoR$^{+2U\ (or\ +5U)\ Epo/ml}$, MCF7, MCF7$^{+2U\ (or\ +5U)\ Epo/ml}$, and MCF7/EpoR^{kd}, MCF7/EpoR$^{kd\ +2U\ Epo/ml}$, MCF7/EpoR$^{kd+5U\ Epo/ml}$ - are powerful tools that will allow us to carry out further studies designed to investigate the role of EpoR (acquisition) in mammary carcinogenesis.

In summary, we have demonstrated that EpoR acquisition by non-tumorigenic MCF10A mammary epithelial cells results in cells that

1) appear morphologically distinct and grow faster in culture,
2) become growth-factor independent and Epo-responsive,
3) become anchorage-independent for growth in soft agar,
4) begin to express the gene for Epo, and
5) when chronically Epo-treated (at physiologically relevant Epo doses) develop increased resistance to paclitaxel.

The data suggest that acquisition of EpoR expression by mammary epithelial cells results in cells with an altered (potentially "pre-malignant"?) phenotype and that the resultant functional Epo-EpoR axis may play a role in the process of malignant transformation.

The data also support the need for continued investigation, and potential alteration, in the clinical use of Epo in (at least some) breast cancer patients. In addition our data support the possible future development of the EpoR on tumors as a therapeutic target.

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Appendix (CV for Laurie Feldman, PhD)

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Laurie Feldman, Ph.D.	Assistant Professor of Medicine (Biochemistry)

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Mount Holyoke College, South Hadley, MA	A.B.	1969-1973	biochemistry
Tufts University School of Medicine, Boston, MA	Ph.D.	1973-1980	biochemistry

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past
3 years and representative earlier publications pertinent to this application. PAGE LIMITATIONS APPLY. DO NOT EXCEED FOUR PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Research and Professional Experience

1981-1983 Postdoctoral Fellow, Department of Microbiology, Boston University School of Medicine, Boston, MA
1983-1986 Research Associate, Department of Biomedical Research, St. Elizabeth's Hospital, Boston, MA
1986-1988 Assistant Investigator, Department of Biomedical Research, St. Elizabeth's Hospital, Boston, MA
1986-1988 Assistant Professor, Department of Medicine, Tufts University School of Medicine, Boston, MA
1988-1990 Instructor in Medicine (Biochemistry), Harvard Medical School, Boston, MA
1988-1996 Principal Investigator, Division of Hematology and Oncology, Department of Medicine, New England Deaconess Hospital, Boston, MA
1991- Assistant Professor of Medicine (Biochemistry), Harvard Medical School, Boston, MA
1996- Principal Investigator, Division of Hematology and Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA
1998- Member, Beth Israel Deaconess Cancer Center, Boston, MA
2003- Member, Dana Farber/Harvard Cancer Center, Boston, MA
2004- FDA Oncology Drug Advisory Committee, consultant

Honors and Awards

1997 Focused Giving Award, Robert Wood Johnson Foundation
1995, 2002 Aid for Cancer Research Equipment Awards

1973 AB, Magna Cum Laude, Mt. Holyoke College
1972 Louisa Stone Stevenson Prize in Chemistry, Mt. Holyoke College
1970 Bernice Maclean Shapiro Prize in Biology, Mt. Holyoke College

Representative Research Publications

1. **Feldman L**, Stollar BD. Serological analysis of the H3-H4 histone complex. Biochem. 1977; 16:2767.
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Abstract for American Association of Cancer Research annual meeting (Los Angeles, CA – April 2007)

Expression of the erythropoietin receptor (EpoR) by mammary epithelial cells results in a premalignant phenotype

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A growing number of in vitro studies have demonstrated the presence of functional erythropoietin receptors (EpoR) on tumor cells and cell lines. In addition, several large clinical trials have indicated an effect of erythropoietin (Epo) on the growth and survival of primary tumors. The data suggest that, in the majority of cases, the normal (i.e. nontumorigenic) counterparts of these tumor cells also express EpoR. The significance of extra-hematopoietic EpoR in general, and their potential role(s) in the growth and survival of cancer cells in particular, are not well understood. One notable exception to the appearance of EpoR on both normal and malignant cells is that of mammary epithelial cells. RT-PCR and immunohistochemical studies have demonstrated that normal mammary epithelial cells do not express EpoR (gene or protein), while breast cancer cells express EpoR at both the gene and protein level. This differential expression of EpoR has led us to hypothesize that acquisition of EpoR may be related to the oncogenic process in breast cancer. To test this hypothesis, we stably transfected (nontumorigenic) MCF10A mammary epithelial cells with a human EpoR cDNA in pcDNA3.1, to derive MCF10A/EpoR cells. "Control" MCF10A cells were transfected with pcDNA3.1 vector alone, and the resultant stable transfectants were designated MCF10A/vector. MCF10A/EpoR (but not MCF10A/vector) have altered morphology in culture, losing their fusiform structure and resembling (tumorigenic) MCF7 cells in appearance. MCF10A/EpoR cells lose their dependence on EGF and insulin; they respond to rhEpo in culture; and they become anchorage independent for growth. MCF10A/EpoR cells form colonies in soft agar, and the colony size/number is increased by rhEpo. Our data suggest that acquisition of EpoR by mammary epithelial cells is, at the very least, permissive for anchorage-independent, growth factor-independent growth of the cells in vitro. Further, the data support our hypothesis that EpoR acquisition by mammary epithelial cells may be part of the process of malignant transformation and may contribute to the oncogenic process.

Abstract # 5286:

Expression of the erythropoietin receptor (EpoR) by mammary epithelial cells results in a premalignant phenotype

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Introduction and hypothesis

A growing number of *in vitro* studies have demonstrated functional erythropoietin receptors (EpoR) on tumor cells and cell lines. Several large clinical trials have indicated an effect of erythropoietin (Epo) on the growth and survival of primary tumors. The significance of extra-hematopoietic EpoR in general, and of their potential role(s) in the growth and survival of cancer cells in particular, are not well understood. Available data suggest that, in the majority of cases, normal/nontumorigenic cell counterparts of EpoR$^+$ tumor cells also express EpoR. One notable exception to the appearance of EpoR on both normal and malignant cells is that of mammary epithelial cells. Both RT-PCR and immunohistochemical studies have demonstrated that normal mammary epithelial cells do not express EpoR, while breast cancer cells express EpoR at both the gene and protein level. This differential expression of EpoR has led us to hypothesize that acquisition of EpoR may be related to the oncogenic process in breast cancer.

Methods and research design

MCF10A mammary epithelial cells were transfected with either the full-length human EpoR cDNA in vector pcDNA3.1 (pcDNA3.1$^+$/EpoR)* or with pcDNA3.1$^+$ vector alone. Stable cell lines were selected for 2 months in 600 µg G418/ml, and the resultant cell lines were designated as MCF10A/EpoR and MCF10A/vector, respectively. MCF10A/EpoR cells were subjected to single-cell cloning, and both cloned and uncloned MCF10A/EpoR cells were used for further analysis, as indicated in the figures that follow.

*Rouleau C, Cui K, Feldman L. Exp. Hematology, 2004; 32: 140-148.

Beth Israel Deaconess Medical Center

Harvard Medical School Teaching Affiliate

Results

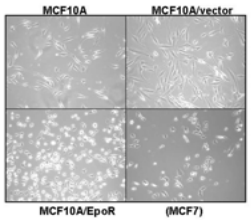

Figure 1: MCF10A/EpoR cells (bottom, left) proliferate more rapidly in culture and undergo morphological changes relative to either "parental" MCF10A or MCF10A/vector (top, left and right, respectively). MCF7 breast cancer cells are shown lower right for comparison.

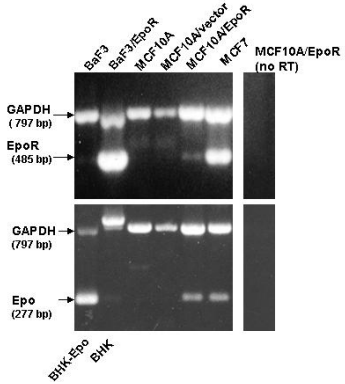

Figure 4: RT-PCR reveals that MCF10A/EpoR cells express the same size EpoR fragment as do MCF7 breast cancer cells (top). Interestingly, cells transfected with EpoR (I.e. MCF10A/EpoR cells) also express the gene for Epo. Neither MCF10A nor MCF10A/vector cells express Epo or EpoR.

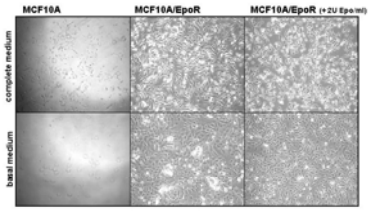

Figure 2: "parental" MCF10A (left) and MCF10A/EpoR cells were maintained either in complete (DMEM/F12 – 5% FBS with EGF, insulin and hydrocortisone; top) or in basal (DMEM/F12 - 5% FBS; bottom) medium. MCF10A cells have an absolute requirement for growth factors for *in vitro* survival and growth, while MCF10A/EpoR proliferate robustly, even in the absence of exogenous growth factors. The addition of 2U rhEpo/ml to basal culture medium further enhanced the growth of MCF10A/EpoR cells (bottom, right).

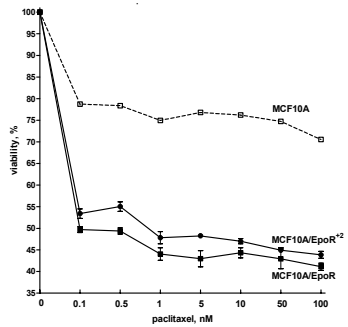

Figure 5: Cells, as indicated, were incubated in the absence or presence of paclitaxel (0 – 100 nM) for 48-hr, and cell viability (survival in presence of given dose paclitaxel ÷ survival in absence of paclitaxel) was measured by an MTT assay. Paclitaxel acts by inhibiting microtubule growth and preferentially affects rapidly-growing cells. Data from a recent preliminary experiment indicate that MCF10A/EpoR cells (■) are sensitive to paclitaxel; and that passage of MCF10A/EpoR cells for 2-mo in the presence of 2UrhEpo/ml results in cells (designated MCF10A/EpoR^{+2}) with slightly increased resistance to paclitaxel (●). By comparison, slow-growing MCF10A mammary epithelial cells (□) have limited sensitivity to paclitaxel over the time of this experiment.

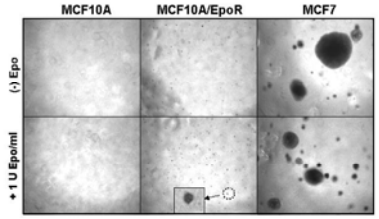

Figure 3: MCF10A cells(left) are anchorage dependent for growth – i.e., they fail to form colonies in soft agar. Conversely, MCF7 breast cancer cells (right) are anchorage independent for *in vitro* growth. MCF10A/EpoR cells (middle) appear to exhibit anchorage-independent growth, forming small colonies in soft agar. The addition of rhEpo to culture of MCF10A/EpoR cells increases the number of soft agar colonies that are formed.

Summary and conclusions

Stable transfection of MCF10A mammary epithelial cells with the full-length EpoR cDNA results in a stable cell line, MCF10A/EpoR, which is phenotypically distinct from parental MCF10A cells.

1. MCF10A/EpoR cells have altered morphology and increased proliferation in culture.
2. MCF10A/EpoR cells are growth factor independent for *in vitro* growth.
3. MCF10A/EpoR cells are anchorage independent and form colonies in soft agar. Growth of these colonies is stimulated by rhEpo.
4. Parental MCF10A cells lack expression of the EpoR or Epo genes, while RT-PCR reveals that MCF10A/EpoR cells express both the EpoR and Epo genes.
5. Chronic low-dose Epo-treatment of MCF10A/EpoR cells results in increased resistance to paclitaxel.

The data suggest that acquisition of EpoR expression by mammary epithelial cells results in cells with an altered phenotype, one more closely resembling that of breast cancer cells. We hypothesize that EpoR acquisition by mammary epithelial cells may play a role in the process of malignant transformation.

This work was supported by DoD BCRP Concept Award W81XWH-05-1-0522 to L.F.